ATTACHMENT C

The two-component sensor kinase gacS is involved in biofilm formation by *Pseudomonas chlororaphis* O6 and *Pseudomonas aeruginosa* PA14

Abstract

The root-colonizing bacterium *Pseudomonas chlororaphis* O6 (*Pc*O6) suppresses fungal pathogens through the production of phenazines and stimulates induced resistance in tobacco against bacterial and viral pathogens. To understand the processes involved in root surface colonization, the potential to produce biofilms was evaluated. Biofilms formed by the wild-type were compared *in vitro,* using the Calgary Biofilm Device, to those produced by a GacS mutant. The GacS mutation eliminated traits displayed by dense populations including the secretion of several secondary products. Biofilms formed by the *gacS* mutant, as evaluated by colony counts and SEM, remained flat and immature compared with the layered and channeled structures formed by the wild type strain under both rich and minimal growth conditions. In addition, a Pseudomonas aeruginosa gacS knockout mutant was evaluated for biofilm formation, showing similar patterns of biofilm formation Complementation of both mutants with an active *gacS* gene restored mature biofilm formation under rich growth conditions. In addition, further analysis of PcO6 gacS mutant showed reduced levels of 3-oxo-C6-AHSL and C8- AHSLs in comparison to wild type and complemented strain. Also, PcO6 gacS knock-out presented increased susceptibility to hydrogen peroxide, in comparison to wild type. The results demonstrate that the

regulatory *gacS* gene plays an important role in the later stages of biofilm formation, affecting the structure of *Pc*O6 biofilms.

Introduction

Fluorescent pseudomonads are competitive and aggressive colonizers of plant roots. Several isolates protect plants against pathogen challenge, through direct antagonism or by activation of plant defense mechanisms. These bacteria form microcolonies on the root surface (Tombolini et al, 1999). How these structures relate to mature structured biofilms is uncertain.

Biofilms are structured communities of microbial aggregates attached to a surface, enclosed in a polymeric matrix permeated by water channels. Biofilms are associated with human infections (McClean, et al., 1997) and plant diseases of citrus and grape caused by the bacterial pathogen *Xylella fastidiosa*. The findings that biofilm cells are less susceptible to antibiotics and host defenses imply that the ability to produce a biofilm may aid the process of colonization. The role of biofilms on host colonization has been demonstrated.

Cells in biofilms are phenotypically distinct from free-living (planktonic) cells (Radtke, et al., 1994). Changes in gene expression associated with sessile growth in fluorescent pseudomonads have been demonstrated. *P. putida* undergoes several physiological changes in the initial hours after attachment to inert surfaces. Phenotypical changes have been detected, through proteomics, throughout different stages of biofilm development in *Pseudomonas aeruginosa*, a classic model for biofilm studies and important human pathogen associated to cystic fibrosis associated pneumonia.

Although some genes have been identified as triggers or regulators of biofilm formation, a global understanding of the process is far from being sketched.

Studies in biofilm formation of *P. aeruginosa* demonstrated that a mutation on a two-component regulator, *gacA*, made the mutant unable to produce biofilms under *in vitro* conditions, in contrast to the wild type. The *GacA* gene (Global Activator of antibiotic and cyanide) is the cognate response transcriptional regulator that interacts with *gacS* (primarily identified

as *lemA* in *P. syringae*), which is similar to the transmembrane histidine kinase portion of the two-component regulatory system (Hrabak & Willis, 1992).

GacA/*GacS* may play different roles depending on the bacterial species. In *P. syringae*, it affects lesion formation in beans, as well as protease, seryngomycin, alginate, AHSLs production and swarming. In *P. fluorescens*, gacA/gacS is involved in cyanide and antibiotic formation, protease and phospholipase production, playing a role in the biocontrol and stress response. GacA/GacS systems also regulate the production of anti-fungal compounds and/or virulence factors in other plant-associated bacteria (Corbell et al., 1995; Hrabak et al., 1992).

The sensor-kinase pair of genes, *gacA-gacS*, also interacts with another major gene regulation system, the quorum-sensing, which coordinate population density-dependent gene expression (Kim et al., 1997). Quorum-sensing, or auto-induction, allows bacteria to detect variations in population density by sensing the increasing concentration of small diffusible molecules such as acyl-homoserine lactones (AHSLs), leading to the expression of specific sets of genes in high cell densities (Kim et al., 1997). It is controversial whether quorum-sensing regulation plays a role in biofilm development, especially after the initial stages of attachment and microcolony formation.

P chlororaphis O6 is also an aggressive root colonizer, and like *Pc* 30-84, the synthesis of phenazines, extracellular protease and HCN are under *gacS* control. Under greenhouse conditions, both the wild type and GacS strains colonize tobacco roots and confer induced resistance in leaves to *Pseudomonas syringae* pv syringae. Phenazines are cited as being important in maintaining field populations of *Pc*30-84 and in antagonizing the growth of fungal pathogens. Cell surface features and CN production also are altered in mutations in the *gac* sensors-kinase genes. In *P. fluorescens*, *gacA/gacS* likewise is involved in cyanide, antibiotic, protease and phospholipase production, thus influencing biocontrol and survival potential.

GacS mutants of *Pc*O6 can still colonize the plant roots, similarly to observations for Gac mutants of *Pc*30-84, but presents reduced fitness under competitive conditions in comparison to the wild type.

These observations prompted our interest in studies on the capacity of Pseudomonas chlororaphis O6 wild-type and gacS mutant to generate biofilms. In parallel, the effect of gacS mutations on biofilm formation in *Pseudomonas aeruginosa* PA14 was also investigated. In this paper, we report on using an in vitro model, the Calgary Biofilm Device (CBD), to investigate the effect of the *gacS* mutation of *Pc*O6 and PA14 *in vitro* biofilm formation. The CBD allows for the formation of 96 equivalent biofilms on polystyrene pegs under shear force and can be used for quantification of biofilm and planktonic growth and also for morphological analysis.

Our goals were to establish the role of gacS in biofilm formation. We expect to: i) begin to elucidate the importance of biofilm formation on root colonization, which may help exploit these bacteria as biocontrol agents for plant diseases; ii) define possible targets for development of strategies for controlling or reducing biofilm infections.

Materials and Methods

Bacterial strains and growth conditions.

Pseudomonas chlororaphis O6 wild type strain was isolated from roots of field-grown wheat (Kropp, et al., 1996). A mutant deficient in *gacS* was generated by insertional mutagenesis with a kanamycin resistance gene as described by. Complemented mutants were derived by insertion of a stable plasmid bearing the wild-type gacS gene, under control of its own promoter, and a tetracycline resistance marker, into the gacS knock-out mutant. Cultures were stored at –70°C in 15% glycerol. Bacteria were grown in: King's medium B (KB) at room temperature (20 - 22°C), with shaking at 120 rpm, or on agar plates at 28°C, supplemented with appropriate antibiotics, kanamycin and tetracycline at 25 mg/mL. Minimal medium included (10.5g K_2HPO_4, 4.5g

KH_2PO_4, Sodium Citrate 0.45g, ammonium sulphate 1.0g, supplemented with MgSO4 and sucrose (Kim et al., 1997). Pseudomonas aeruginosa PA 14 strain was grown in LB media. Mutant was grown in LB supplemented with Kanamycin.

Biofilm formation and planktonic counts Biofilms were cultured on the Calgary Biofilm Device (CBD), in KB broth and in minimal medium for P. chlororaphis and LB broth for P. aeruginosa strains, using procedures published by Ceri et al. (1999, 2001). Briefly, the device features a microtiter plate lid with 96 polystyrene pegs or protrusions distributed on the lid. The pegs fit precisely into the wells of a standard 96-well microtiter plate. This 96-peg plate lid fits over a special bottom trough, which contains the microbial inoculum, and placing the device on a rocking platform that allows an equivalent shear force to be created on all 96 biofilms growing on the pegs.

The plates were prepared as follows. Inocula were prepared in PBS, with turbidity to match a McFarland Standard of 1.0 (3.0×10^8 cfu/mL), starting from a overnight liquid culture. This suspension was diluted 1:30 V/V in appropriate broth and planktonic and biofilm growth were started by adding 24 mL of each inocula into the CBD trough. The trough were then covered by the 96-peg lid and the device was placed on a rocker (Red Rocker, Hoefer Instruments, 10 rpm) in a 28°C incubator, with the troughs parallel to the direction of motion of the rocker. The planktonic bacteria grew in the broth sitting in the bottom trough, simultaneously with the biofilms formed on the pegs.

Modifications to the method involved sonication of the sampled pegs at defined times in PBS-Tween buffer (0.2 M phosphate-buffered saline (PBS), pH 7.2 plus 1% Tween 20 v/v), for 30 min. at high intensity, using an Aquasonic sonicator (model 250HT, VWR Scientific). Culturable colonies were determined by spot plating (plating of 20 μL of each sample's dilution on appropriate agar plates, to yield isolated colonies to be counted as colony forming units [cfu]).

Experiments with minimal media were started from 24-36h liquid cultures in minimal media, using the same procedure described above. The minimal growth of PcO6 was measured by fluorescent direct counts.

Total direct counts: Total direct counts were performed on bacteria grown on minimal media and were based on methods described in. At each time point, 1.0 mL of planktonic bacteria was centrifuged for 10 min. and resuspended in PBS. This step was repeated. The bacterial suspensions were stained with DNA-binding fluorescent dyes Live-Dead staining kit - according to manufacturer's protocol (Molecular Probe).

The suspensions of the stained cells were filtered through 25mm 0.2 μm black polycarbonate membranes (Nuclepore Track-Etch, Whatman), under vacuum (20 tor). Samples were fixed by passage (1 ml/filter) of 5% glutaraldehyde in sodium cacodylate buffer, pH 7.2. Membrane filters were allowed to dry, mounted and fluorescent cells were counted using a Nikon Labophot epifluorescence microscope (Japan), equipped with an HBO 100 W light source. Ten to thirty randomly selected microscopic fields were counted. Cell numbers/ml were calculated following the formula:

B = (N/X) (A/B) (1/S), where N = number of bacteria counted, X = number of fields of view (grids), A = area covered by sample), B = area of the field of view and S = the amount of sample on the slide (volume). The number will reflect the cfu/mL, which multiplied by the original volume of sample give an estimation of total cfu.

Statistical analyses: Plate counts were analyzed with a one-way analysis of variance (ANOVA) table using Prism 2.1 software. Significance of difference between groups was determined using the Newman-Keuls Multiple Comparison test with a confidence interval of 95%.

Scanning Electron Microscopy

After 10, 24 and 48 h, pegs were removed from the 96-peg lid of the MBEC device and air dried for 1-2 h at room temperature, under a fume hood. Samples were fixed at room temperature, in 5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, pegs were allowed to dry overnight on a Petri dish, then assembled onto stubs and sputter-coated with gold-palladium. Scanning electron microscopy was performed by using a Cambridge Model 360 SEM at 20 Kv emission. Digital images were captured from the SEM using OmniVision (v. 5.1) software and imported into Adobe Photoshop 6.0 (Windows v. 6.0; Adobe Systems Incorporated, San Jose, CA, USA) for figure composition and printing. Data shown are representative of hundreds of fields of view for each treatment. Treatments and SEM analysis have been repeated at least 3 times. At least three sampled pegs of each strain have been analyzed at each time.

Attachment and microcolony formation. The ability to of bacteria attach to the polysterene pegs and to form monolayered microcolonies was evaluated by analysis of the electron micrographs.

Determination of minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC)

The Minimum Inhibitory Concentration (MIC), which represents the concentration of antibiotic required to inhibit growth of a planktonic bacterial population, was determined using the Calgary Biofilm Device. This method generates MIC values equivalent to that obtained using the National Committee for Clinical Laboratory Standards (NCCLS) procedure.

Assays were performed when pegs contained 10^5 - 10^7 bacteria growing as a biofilm, following conditions described above for the CBD and estimated established from growth curves. Each test antibiotic was placed in one lane of the microtiter plate at 2 fold dilutions of antibiotic (from 1024 µg/ml to 1 µg/ml). Non-adherent bacteria on the pegs were washed previously in a 96 well microtiter plate containing sterile PBS. The lids containing the pegs with the bacterial biofilms were then secured over the test microtiter plates containing the antibiotic solutions and the plates were incubated for 24 hours at 28°C for PcO6 strains and 37°C for Pa14. The lid was removed and the MIC was determined from the bacteria that were shed from the biofilms on the pegs into the different concentrations of antibiotic solutions in the 96 well plates (Ceri et al., 1999; 2001).

The lid was rinsed in PBS, and placed over a second 96 well microtiter plate containing fresh sterile broth medium. The remaining biofilm was removed from the pegs by ultrasonic disruption for 5 minutes. The plates were incubated once more for 24 hours at 28°C for PcO6 strains and 37 °C for Pa14 37 °C and the presence of viable bacteria was determined by turbidity determined at 650 ηm in a 96-well plate reader (Molecular Devices, Fisher Scientific). Growth of bacteria in a particular well indicates re-growth of planktonic bacteria from surviving biofilm. Therefore the MBEC value represents the lowest dilution at which bacteria fail re-grow. Twelve antibiotics were evaluated (six on each plate) and two lanes served as a negative control (no antibiotic).

AHSL production: The HSL components produced by O6 were extracted in 50 ml of ethyl acetate from 8, 16 and 24 h cultures grown in 50 ml of KB

medium. The ethyl acetate was evaporated at room temperature and the residue dissolved in 1 ml ethanol. 10 µl aliquots were separated by TLC and the presence of the HSLs detected by restoration of pigmentation to a mutant indicator strain of *Agrobacterium tumefasciens* that lacks synthesis of its own HSL (McClean et al., 1997).

Results

GacS mutant characterization – Biofilm formation

Biofilm formation by wild type, a gacS mutant and a complemented strain of *Pseudomonas chlororaphis* O6 (PcO6) and *Pseudomonas aeruginosa* PA14 (PA14) were characterized using the CBD as the *in vitro* model. The results showed no differences in planktonic growth among PcO6 strains in KB broth, or PA 14 strains in LB broth. On the other hand, the *gacS* mutant presented a 10 to 15 fold reduced biofilm growth in comparison to the wild type and complemented strains, for both PcO6 and PA14. Wild type and complemented strain biofilm formed comparable biofilms on the CDB (Figure 1A and 1B).

Extensive scanning electron microscopy analyses were performed on biofilms sampled at 24h and 48 h of growth for the PcO6 strains. Based on SEM analysis, no differences in attachment or formation of microcolonies was observed between the wild type, mutant and complemented strains on the polystyrene surface of the CDB (Table 1).

However, the gacS mutant failed to form mature biofilms (Figure 2). The biofilm structure has been assessed by SEM and the pictures shown in Figure 2 are representative of hundreds of micrographs. Biofilms formed by

wt were over 10 μm thick after 48 h, with cells arranged contiguously, compacted, in very extensive biofilms, in contrast with the observed for the gacS mutant. In the mutant, mostly monolayers or small, reticulated patches of microcolonies were registered, with no observation of mature biofilms and no coverage of large extents on the surface. The complemented strain was very similar to wild type. The whole coverage area on the peg's surface was reduced compared with the almos complete coverage of the wild type.

By 48 h the O6 wild type strain were over 10 μm thick with cells arranged in a very organized fashion showing layering and structured channels. The biofilms formed by the complemented strain were very similar to wild type. However, the *gacS* mutant failed to form such mature biofilms (Figure 2). At 48 h, biofilms of the *gacS* mutant were still mostly of monolayers with only small patches of microcolonies.

Minimal Media

When the strains were grown in minimal media, differences between biofilm growth between wild type and gacS mutant took longer to be detectable. Growth up to 48 h did not show a significant difference. However, there is evidence of occurrence of viable but non-culturable (VBNC) cells in all cultures tested, since the curves obtained by viable plating and live-dead staining fluorescent counts differed among various experiments. Other major PcO6 characteristics such as phenazine production were also delayed in minimal media. In rich media, phenazine production is usually visible at about or shortly after 24 h, but in minimal media it took longer than 48h.

There are indications that the smaller difference in cell numbers in biofilms formed in minimal media between wt and gacS mutant were due to a delay in biofilm formation by the wt and not due to enhancement of biofilm formation by gacS mutant. After 48h, a thin biofilm starts to show for wt strain whereas the gacS mutant continues to show no development of mature biofilm. Growth of the wild type strain was slower on the minimal than the KB medium. The GacS planktonic cells grew at a more similar rate to the wild type. SEM analysis revealed that, by 48h, a thin biofilm was forming for the wild-type strain whereas none was apparent for the *gacS* mutant.

Assessment of plank tonic and sessile cells growing in minimal media showed large differences between estimated cfu by plating techniques in comparison to direct fluorescent counts.

Attachment and motility: Based on SEM analysis, no differences in attachment or formation of microcolonies was observed between the wild type, mutant and complemented strains on the polystyrene surface of the CDB (Table 1).

Twitching, swarming and swimming abilities of mutant, wild type and complemented strains have been assessed. There was no significant difference between the wild type, gacS mutant and complemented mutant strains when assessed for different type of motility (Table 1).

Antibiotic Susceptibility tests:

In addition to the growth and morphological analysis, the strains were tested for susceptibility to antimicrobials, to evaluate if biofilm formation by PcO6 would confer any enhancement in resistance against antimicrobial compounds. Minimal inhibitory concentration (MIC) and Minimal Erradication Biofilm Concentration (MBEC) of different classes of antibiotics were assessed against planktonic and biofilm populations of PcO6 wt, gacS mutant and gacS + using the CBD. The. This device allows for multiple biofilms to be assayed at once by placing it on a regular 96 well plate containing different concentrations of antibiotic and biocides.

An initial screen of 12 antibiotics indicated that resistance of strains was enhanced in biofilms in comparison to planktonics, for the wild type, gacS mutant and complemented strains. The antibiotics tested and resistance level are presented in Table 2. All strains showed same level of resistance against beta-lactam antibiotics (group 1), but resistance to aminoglycosides were shown to vary among the strains.

GacS affects biofilm formation in a later stage of biofilm development.

GacS mutant has been demonstrated to be defective in phenazine and hydrogen cyanide production, characteristics that may interfere with their biocontrol capacity. On the other hand, the mutant is enhanced in its capacity to produce siderohpores.

Discussion

AHSLs are only partially responsible for phenotypic changes associated to surface attachment in P putida. Although the knowledge on the first stages of biofilm development, i.e., transport and attachment to a surface and subsequent formation of microcolonies has expanded in the past few years, an understanding the steps between microcolony and mature biofilm formation is still beginning. In this paper, a mutation in gacS seem to affect steps in biofilm formation other than attachment, motility of microcolony formation.

Contrary to observed by Chancey et al (2002), showing that gacS mutant enter exponential growth faster than the wild type, we found no differences in planktonic growth between gacS mutant and wt of P. chlororaphis O6 in rich media (KB).

Chancey et al observed, through comparisons of wt x mutant populations in sterile and natural soil, that a functional gacS system confers a survival advantage, especially in the presence of indigenous microflora. In addition, experiments with mixed populations of wt and mutant strains suggested that there is no displacement of wild-type populations and there is enhanced survival of the wt in the presence of mutants, suggesting that occurrence of gacS mutants may be a normal and beneficial component of rhizosphere populations of P. aerofasciens.

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Table 1. Characteristics of gacS knock-out mutants, wild type strains and complemented mutant strains

	Wt	GacS-	GacS/+
AHSL – C4	+++	-	+++
Siderophores	-	+++	-
Attachment KB	+++	+++	+++
Attachment Min. Media	+++	+++	+++
Microcolony	+++	++	+++
Microcolony Min. Media	+++	+	++
Swarming	+++	+++	+++
Swimming	+++	+++	+++
Twitching	+++	+++	+++
Biofilm formation KB	+++	-	+++
Biofilm –Min. Media	++	-	++

Table 2 : MICs and MBECs of PcO6

Antibiotic	class	PcO6		gacS mutant		gacS +	
		MIC(mg)	MBEC	MIC	MBEC	MIC	MBEC
Ampicillin	Beta-lactam	> 1024	> 1024	> 1024	> 1024	> 1024	> 1024
Ceftazidine	Cephalosporin	> 1024	> 1024	> 1024	> 1024	> 1024	> 1024
Cyclohexamide	Beta lactam	> 1024	> 1024	> 1024	> 1024	> 1024	> 1024
Piperacillin	Beta lactam	> 1024	> 1024	> 1024	> 1024	> 1024	> 1024
Erythromycin		512	> 1024	512-1024	>1024	512	>1024
Oxy-tetracyclin	Tetracyclin	512	>1024	1024	>1024	1024, > 1024	>1024
Polymixin-B		32, 128	256	64	256, 128	64, 32	128 , 256
Spectinomycin		32, 128	>1024	1024	1024, >1024	128, 256	>1024
Gentamycin		16	1024, >1024	64	>1024	16, 32	1024
Streptomycin		512, 1024	>1024	1024	>1024	512, 512	> 1024
Tobramycin		8	512, >1024	16	256, 1024	8, 32	>1024

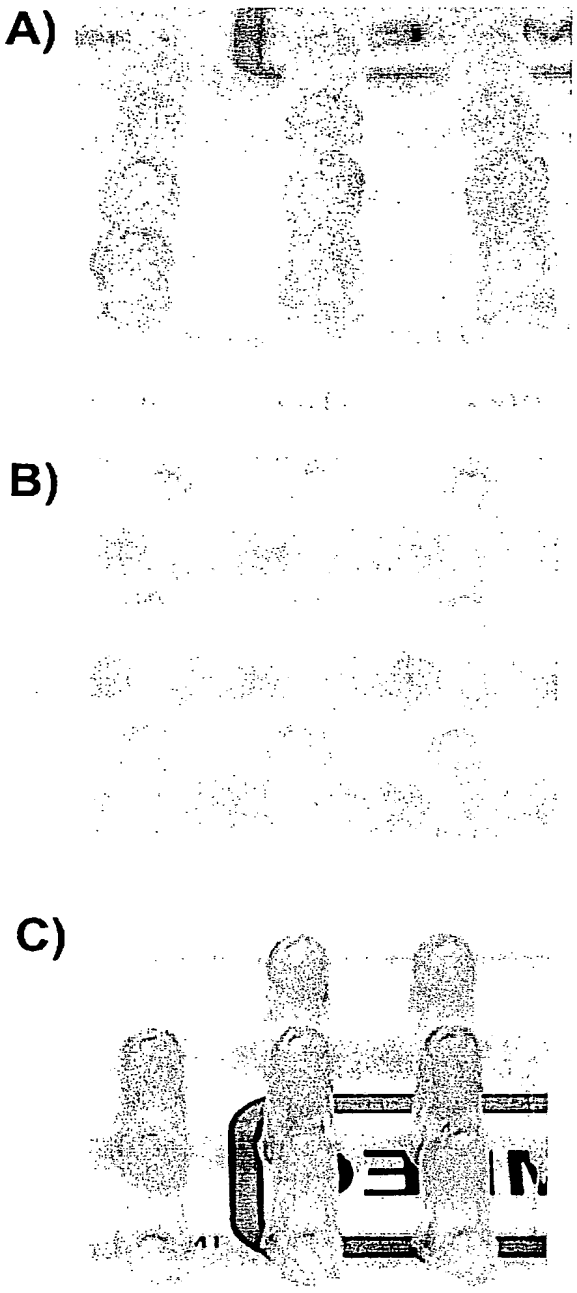

Figure 1. Pseudomonas chlororaphis O6 biofilms formed on MBEC device, showing decrease in biofilm formation by a mutant defincient in gacS gene. A) wild type strain; B) gacS mutant; C) gacS mutant complemented with gacS gene.

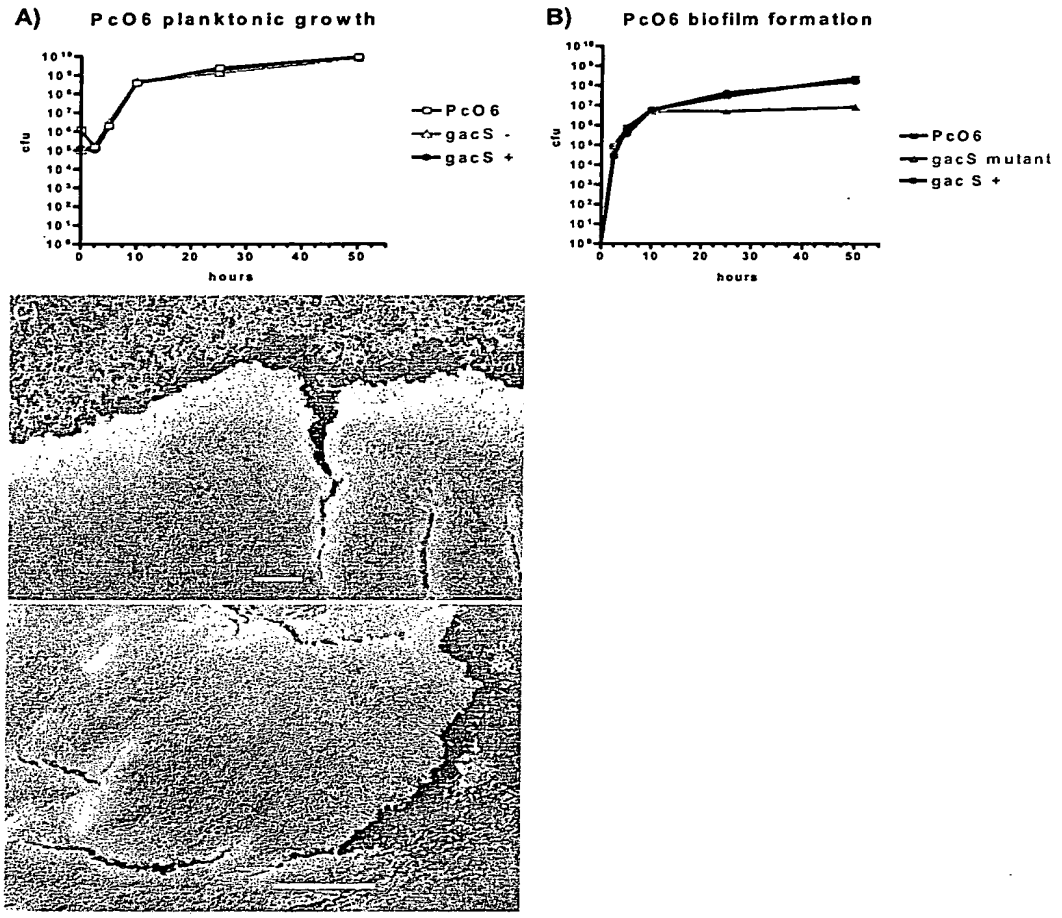

Figure 2 : *In vitro* biofilm formation by *P. chlororaphis* O6 wild-type and GacS mutant. A) Planktonic growth showing no difference between O6, GacS mutant and GacS+ complemented mutant; B) Biofilm growth of same strains, showing reduced biofilm mass by GacS mutant (restored to wild type levels by complementation with gacS construct); C) , D) - Scanning micrographs showing 24h biofilms of O6 wt (C) and GacS mutant (D); and E) , F) 48h biofilms of O6 wt (E) and gacS mutant (F), outlining the structural differences observed between biofilms formed by the strains. Note the flat, monolayered structure formed by the gacS strain, covering the surface in scattered patches (D, F), as opposed to the thicker, multi-layered, more three-dimensional biofilms formed by wt O6 (C,E).

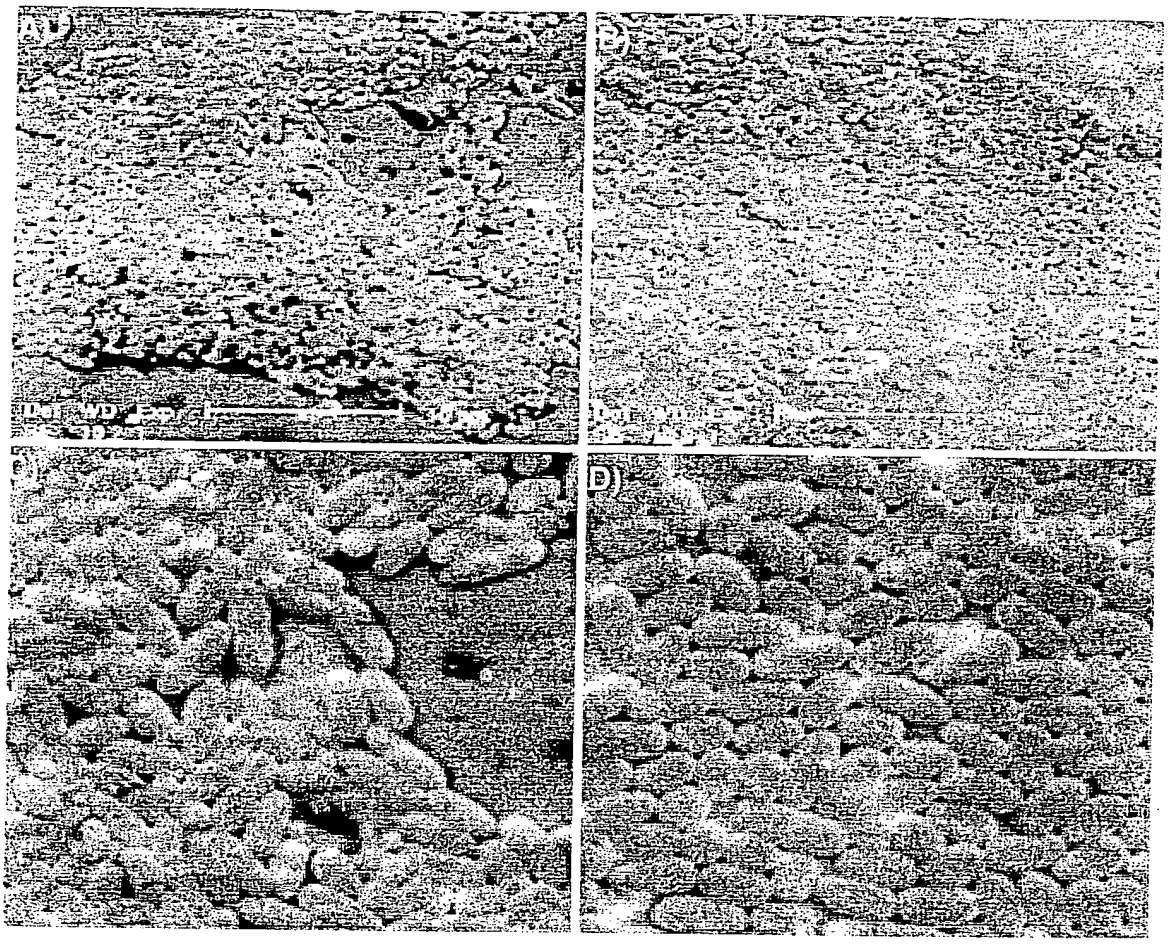

Figure 3.. PcO6 strains biofilms formed on polystyrene surface, in minimal media, after 24h. A) wild type; B) gacS mutant; C) and D) higher magnification of A and B, respectively.